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Quantification, Antioxidant and Free Radical Scavenging Potentials of Polyphenols from Crude Extracts of *Phyllanthus amarus* leaves

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ABSTRACT

This work examined the quantity of Polyphenols (tannins, flavonoids and phenolic acids) and their antioxidant and radical scavenging activities in five solvent extracts of *P. amarus* leaf using assay methods such as DPPH, ABTS, OH, NO and FRAP. Solvents of varying polarity (hexane, chloroform ethyl acetate, acetone, methanol and aqueous) were used for extraction. Results revealed various extraction yields; the highest was aqueous, methanol and ethyl acetate in this order compared to acetone, chloroform and hexane, indicating that extraction efficiency favors high polar solvents. The solvents extract various phytochemicals with ethyl acetate, aqueous and methanol showed better phytochemical distribution. The result also showed that, acetone extract has more of flavonoids followed by ethyl acetate; the least was found to be hexane and chloroform extract while tannin and phenolic acids were higher in ethyl acetate extract. Investigating the fifty percent inhibitory concentration (IC_{50}) of the tested extracts demonstrated that, the ethyl acetate extract which generally gave the highest percentage inhibition, showed the lowest IC_{50} for ABTS, NO and OH at p=0.05. The correlation between IC_{50} values and polyphenol content were analyzed for each extract using Pearson's correlation analysis. It was found that the ethyl acetate extract showed positive and significant correlation between $ON^{\cdot}$, ABTS, FRAP and phenolic acids with coefficient of 0.993, 0.996, and 0.920 respectively and OH radical to flavonoids and tannins with coefficient of 0.875 and 0.866 respectively. DPPH to tannins has a coefficient of 0.854. An evaluation on the correlation analysis revealed that ethyl acetate extract is the best. Conclusively, the richer the polyphenol content, the better the antioxidant potential of plant and ethyl acetate is the best solvent for extraction of polyphenols to address diseases that relate to oxidative stress.

Keywords: Antioxidant, Correlation study, *Phyllanthus amarus*, Polyphenols

INTRODUCTION

Plants act as hydroxyl (OH) and nitric oxide (NO) radicals' stabilizer through hydrogen or electron donation because of their rich polyphenol contents with strong antioxidant property (Erkan *et al*., 2011; Jian-Ming *et al*., 2010). Antioxidants are either natural enzymatic or non-enzymatic compounds, which may be endogenic, or exogenic to the body but endogenic to plant; and act as defense system in living cells. They are involved in xenobiotic metabolisms for easy elimination of toxic products from the body system. And as natural plant antioxidants, flavonoids, alkaloids, tannins, phenols, stilbenes and terpenoids are used by plants as behavioral defense against inner and external unfavorable environmental conditions (Moukette *et al*., 2015).

Several assay methods are used to screen and confer antioxidant potential of plants and sometimes to understand the probable mechanism of action of antioxidants (Antolovich *et al.*, 2002). This help in understanding the role in minimizing the oxidative stress linked to pathophysiology of diseases. Those commonly used are beta-carotene or crocin bleaching assay, Oxygen Radical Absorbance Capacity (ORAC), Inhibited Oxygen Up-take (IOU), Lipid Peroxidation Inhibition Capacity (LPIC) assays, Total Radical Trapping Antioxidant Parameter (TRAP), Copper Reduction Assays (CRA), Ferric Reducing Antioxidant Power (FRAP) assays, total phenolic content assays by Folin-Ciocalteu reagents, ABTS (2,2- azinobis-3-ethyl-benzothiazoline-6-sulphonic acid) assays, Nitric oxide (NO) scavenging activity, Plant Hydroxyl ($^{\cdot}OH$) radical scavenging activity and DDPH (2,2-Diphenyl-1-Picryhydrazyl) assays (Prior *et al*., 2005).

Phyllanthus amarus is one of the preferred traditional herbs speculated to be effective in treating liver diseases (Liu, 2003). It is important to have sufficient scientific investigation to the claims that, disease prevention and therapy may be achieved by the use of native plants due to their phytochemicals, thought to boost endogenous

antioxidant defences of the body. In a bite to achieve this, the object of this work was to quantify the polyphenol contents, and determine the antioxidant and free radical scavenging activities of various crude extracts of *Phyllanthus amarus* leaves.

MATERIALS AND METHODS

Collection of Plant Materials

The leaves of *Phyllanthus amarus* were collected from Mkar community in Gboko Local Government area, Benue State, Nigeria and were identified at the Centre for Ethno-medicine and Drugs Development, a subsidiary of Bioresources Development and Conservation Program (BCDP), Nsukka, Enugu state. The leaves were dried at room temperature for two weeks under shade to prevent ultraviolet rays from inactivating the chemical constituents.

Preparation of the Plant Extracts

The extraction was carried out using maceration method. Hence, 40g was weighed and soaked in 400ml each of distilled water, chloroform, acetone, hexane, ethanol and ethyl acetate. The mixtures were shaken for 10 minutes and allowed to stand in a stopped container for 72 hours. The filtrates were dried using water bath at 50^oC in order to obtain the respective crude extracts.

Qualitative Phytochemical Screening of the Plant Extracts

Each of the solvent extract was subjected to qualitative tests for identification of various phytochemicals-tannins, saponins, flavonoids, terpenoids, glycosides, alkaloids, phenolic compounds, steroids and anthraquinones by the methods of Sofowora (2008).

Quantitative Determination of Phytochemicals

Quantification was carried out on each solvent extract using standard methods.

Alkaloids

Exactly 200ml of 10% acetic acid in ethanol was added to each extract in 250ml beaker and allowed to stand for 4 hours. The extract was concentrated on a water bath to one-quarter of the original volume followed by addition of 15 drops of concentrated ammonium hydroxide drop wise until the precipitation was completed, the supernatant was discarded and the precipitates was washed with 20ml of 0.1M of ammonium hydroxide and filtered using whatman filter paper. The residue was dried in an oven at 30^oC, weighed using electric weighing balance, and the percentage of alkaloid was calculated using equation 1 (Chukwuma and Chigozie, 2016).

$$\% \text{ Alkaloid} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample}} \text{x } 100 \quad (1)$$

Flavonoids

Exactly 50ml of 80% aqueous methanol was added to the sample in 250ml beaker, covered and allowed to stand for 24 hours at room temperature. After discarding the supernatant, the residue was re-extracted (three times) with the same volume of ethanol. Whatman filter paper was used to filter the whole solution. The filtrate was transferred into a crucible and evaporated to dryness over a water bath. The content in the crucible was cooled in desiccators and weighed until constant weight was obtained. The percentage flavonoid was determined using equation 2 (Ejikeme *et al.*, 2014; Boham and Kocipai, 1994).

$$\% \text{ Flavonoids} \frac{\text{Weight of flavonoids}}{\text{Weight of sample}} \text{x } 100 \quad (2)$$

Phenols

Defatting of plant sample was carried out for 2 hours in 100ml of ether using a soxhlet apparatus. The defatted sample was boiled for 15minutes with 50ml of ether. Exactly 10ml of distilled water, 2ml of 0.1N ammonium hydroxide solution and 5ml of concentrated amyl alcohol were added to 5ml of the extract and left to react for 30minutes for color development. Separately, 0.20g of tannic acid was dissolved in distilled water and diluted to 200ml mark to furnish the standard which served as the control. Varying concentrations (0.2-1.0g/ml) of the standard tannic acid solution were pipette into five different test tubes to which 2ml of amyl alcohol and 10ml of water were added. The solution was made up to 100ml volume and left to react for 30minutes for color development. The optical density was recorded at 505nm. The amount of phenols in the sample extract was determined by extrapolating from the standard calibration curve obtained from the tannic acid solution.

Tannins

Fifty grams of sodium tungstate (Na_2WO_4) was dissolved of distilled water (37ml), then 10g of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) and 25ml of orthophosphoric acid (H_3PO_4) were added into the mixture and heated for 2 hours, cooled and diluted with distilled water (500ml) to afford Foli-Denis reagent. To 1g of each sample in a conical flask, 100mls of distilled water was added. This was boiled gently for 1 hour on an electric hot plate and filtered using No 1 Whatman filter paper in 100ml volumetric flask. Then, 5.0ml Folin-Denis reagent, 10mls of saturated Na_2CO_3 solution, 50mls of distilled water and 10mls of dilute extract (aliquot) were added into 100mls conical flask for color development. The solution was allowed to stand for 30minutes in a water bath at a room temperature with thorough agitation. With the aid of a Spectrum Lab 23A spectrophotometer, optical density was measured at 700nm and compared with the standard tannic acid

curve. The tannic acid solution was prepared by dissolving 0.20g of tannic acid in distilled water (200ml). Varying concentrations (0.2-10mg/ml) of the standard tannic acid solution were pipette into five different test tubes to which Folin-Denis reagent (5ml) and saturated Na_2CO_3 (10ml) solution were added and made up to the 100ml mark with distilled water. The solution was left to stand for 30 minutes in a water bath at room temperature after which absorbance taken was plotted against tannic acid concentration to obtain a cure (Ejikeme *et al*., 2014; Amadi *et al*. 2004). Equation 3 was used in the calculation;

$$\text{Tannic acid } \left(\frac{\text{mg}}{100\text{g}}\right) = \frac{\text{C x Extract volume x 100}}{\text{Aliquot volume x weight of sample}} \qquad (3)$$

Where C is concentration of tannic acid read off the graph

***In vitro* Antioxidant Assay on Crude Extracts of *P. amarus* Leaves**

Crude extracts of the plant leaves were screened for antioxidant activity using 1, 1-dipheny-2-picryl hydroxyl (DPPH), 2, 2-azinobis-3-ethylbenothiozoline-6-sulfonic acid (ABTS), Hydroxyl radical scavenging, Nitric oxide (NO) inhibition activity and Ferric Reducing Antioxidant Power (FRAP).

DPPH Radical scavenging Activity

Sample solutions at various concentrations (25, 50, 75, 150 and 300µg/ml in ethanol) was added to 1ml of DPPH solution (0.2mM in ethanol) and allowed to stand for 30mins for complete reaction at room temperature, the absorbencies of the solutions were measured at 517 nm. The free radical scavenging activity of each sample was determined by comparing its absorbance with that of a blank solution (no sample). The same procedure was used for vitamin C taking as standard. The ability to scavenge the DPPH radical was determined using equation 4 (Tailor and Goyal, 2014).

$$\text{DPPH scavenging activity (\%)} = \frac{(A_o - A_1)}{A_o} \text{ x } 100 \qquad (4)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

ABTS radical scavenging activity

The $ABTS^+$ cation radical was produced by mixing 14mM ABTS solution (5ml) and 5 ml of 4.9mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 hours. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734nm. The plant extract at various concentrations (25, 50, 75, 150 and 300) with 1 ml of ABTS solution was homogenized and absorbance recorded at 734nm. Ethanol blanks was run in each assay and all measurements were done after 6 minutes. Similarly, the reaction mixture of the standard solution was obtained by mixing 950µl of $ABTS^+$ solution and 50µl of BHT (Butylated hydroxytoluene). The inhibition percentage of ABTS radical was calculated using equation 5 (Re *et al*., 1999).

$$\text{ABTS scavenging activity (\%)} = \frac{(A_o - A_1)}{A_o} \text{ x } 100 \qquad (5)$$

Where A_o is the absorbance of the control and A_1 is the absorbance of the sample.

Hydroxyl radical (HO) scavenging activity

To 1.5ml of the plant extract at various concentrations (25, 50, 75, 150 and 300), 60µl of $FeCl_3$ (1mM), 90uL of 1,10-phenanthrolin (1mM), 2.4mL of phosphate buffer (0.2M; pH 7.8) and 150µl of H_2O_2 (0.17 M) were added. The mixture was homogenized using vortex and incubated at room temperature for 5 mins. Absorbance was read at 560nm against blank and the HO scavenging activity was calculated from equation (6) (Yu, *et al.*, 2004).

$$\text{HO scavenging activity (\%)} = \frac{(A_o - A_1)}{A_o} \text{ x } 100 \qquad (6)$$

Where A_o is the absorbance of the absorbance of the blank and A_1 is the absorbance of the sample.

Nitric oxide(NO) scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide (NO), which interacts with oxygen to produce nitrite ions. Scavengers of NO compete with oxygen, leading to reduce production of NO and a pink colored chromophore formed. The absorbance of these solutions was measured at 540nm against the corresponding blank solutions (Fadzai *et al*., 2014). Percentage inhibition was calculated using equation 7.

$$\text{NO scavenging activity (\%)} = \frac{(A_o - A_1)}{A_o} \text{ x } 100 \qquad (7)$$

Where A_o is the absorbance of the control and A_1 is the absorbance of the sample.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP reagent was generated by mixing 300mM sodium acetate buffer (pH 3.6),

10.0mM (tripyridyltriazine) TPTZ solution and 20.0mM $FeCl_3.6H_2O$ solution in a ratio of 10:1:1 in volume. Samples at different concentrations (25, 50, 75, 150 and 300µg/ml) was added to 3ml of FRAP reagent and the reaction mixture incubated at 37^oC for 30 min. The increase in absorbance at 593nm was measured. Fresh working solution of $FeSO_4$ was used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalent per gram of sample (DW) (Benzie and Strain, 1996).

Statistical Analysis of Data

Data obtained was expressed as mean ± SD of triplicate assays analyzed using one-way Analysis of Variance (ANOVA) and student's t-test where applicable on Statistical Package for Social Sciences (SPSS version 20, Chicago, IL, USA). The IC_{50} values were determined by log probit analysis after performing Duncan Test. Microsoft excels was used for the Pearson's Correlation Analysis. Values at P=0.05 were regarded as significant in comparison with appropriate controls.

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical analysis revealed the presence of various phytochemicals. The detailed assessment and comparison between the different organic solvents are arranged in order of polarity as shown in Table 1. The yields of extracts per solvent in 40g/400ml of the sample are also presented in the table.

The presence of tannins, flavonoids, saponins, steroids, terpenoids, cardiac glycosides, anthraquinones and alkaloids in *P. amarus* leaves as revealed in (Table 1) suggests its usage for various medicinal purposes in folk medicine. Most phytochemicals serve as natural antibiotics, which assist the body system in fighting microbial invasion and infections (Lillehoj *et al*., 2018). Flavonoids for instance, are known to have antioxidant effects and have shown to inhibit the initiation, promotion and progression of tumors (Batra and Sharma, 2013); this is done by either scavenging or quenching free radicals or by inhibiting enzymatic systems responsible for free radical generation (Deepak *et al*., 2015). Thus, as an antioxidant present in this plant suggest its use as hepatoprotective, nephroprotective, antimicrobial, anti-inflammatory and anti-carcinogenic (Kassuya *et al*., 2003 and Adeneye *et al*., 2006). Alkaloids may be responsible for its anti-malarial, analgesic properties and stomach disorder (Okwu and Josiah 2006). Many alkaloids such as morphine, codeine, sanguinarine etc are known for their psychotropic and stimulant, antihyperglycemic, antibacterial, antimalarial and anticancer activities (Kaisa *et al*., 2011 and Noureddine, 2018). While tannins have been reported to possess astringent properties, which hastens the healing of wounds (Okwuonu *et al*., 2017).

Table 1 Phytochemical Screening of Crude Extracts of *P. amarus* Leaves

Phytochemical	Hexane	Chloroform	Ethyl acetate	Acetone	Methanol	Aqueous
Tannins	-	+	++	++	++	++
Saponins	-	-	+	-	-	++
Flavonoids	++	++	++	++	++	++
Terpenoids	++	++	++	+	++	++
Glycosides	+	+	++	++	++	++
Alkaloids	-	-	++	++	+	+
Phenols	-	-	++	-	++	++
Steroids	++	++	++	-	++	-
Anthraquinones	-	-	++	++	-	++
Yield (40g/400ml)	1.30±0.24	1.80±0.56	4.10±1.01	2.40±0.93	5.80±0.32	7.50±1.02

NB: +: present, ++: much present, -: absent

The phytochemical screening revealed the absence of tannins, saponins, alkaloids, phenolic compounds, and anthraquinones in hexane and chloroform extracts, except tannins, which were detected in the chloroform extract. Saponins were absent in all the extracts, but present in the ethyl acetate and aqueous. The solvents used were able to extract various phytochemicals, however ethyl acetate, aqueous and methanol extracts showed a better distribution of the phytochemicals. The extracting yield follow the order of aqueous > methanol > ethyl acetate > acetone > chloroform > hexane extract.

Quantification of Polyphenols in the Crude Extracts of *P. amarus* Leaves.

The quantification of total polyphenols in different solvent crude extracts of *P. amarus* leaves revealed that, acetone extract has more of flavonoids followed by ethyl acetate; the least in

flavonoids is hexane and chloroform. Tannin and phenolic acids were highest in ethyl acetate crude extract (Table 2). The quantitative assessment results show that, the ethyl acetate extract contains the highest amount of tannins (56.63mg/100g), phenols (6.08mg/100g), alkaloids (96.72%/5g), and flavonoids (70.02%/g) than the other extracts.

Table 2 Polyphenol contents in Crude Extracts of *P. amarus* Leaves

Extract	Flavonoids (mg/g)	Tannin(mg/g)	Phenolic acids (mg/g)
Hexane	20.35±0.57[a]	0.00±0.00 [b]	0.00±0.00 [a]
Chloroform	20.35±0.57 [a]	15.33±0.11 [b]	0.00±0.00 [a]
Ethyl acetate	70.02±0.03[b]	56.63±0.29 [b]	6.08±0.01 [b]
Acetone	88.02±1.00 [b]	11.40±0.02 [b]	0.09±0.01 [b]
Methanol	30.35±0.57 [b]	6.68±0.01 [b]	0.60±0.01 [b]
Aqueous	24.99±0.02 [b]	17.67±0.02 [b]	0.86±0.01 [b]

Values are expressed as mean ±SD of three replicates. [a] not significant as compared within column at p=0.05. [b] Significant at p=0.05 as compared within column.

Antioxidant Assay of Crude Extracts from *P. amarus* Leaves

According to Moukette *et al*., (2015), the use of biochemical assays to measure the antioxidant power of plants has become the best reliable and readily available methods. There are several assay methods and it is important to use more than one method because of the variable response engendered by a specific antioxidant in various testing systems in order to understand the mechanism of action of the bioactive component involved (Bhakta and Siva, 2012).

It is now a common knowledge that natural plant antioxidants are better alternative as food additives to synthetic ones such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) due to their susceptibility to quality deterioration in food sequel to their highly volatility at elevated temperature, and negative health effects (Anbudhasan, 2014). This gives more credit to research of this nature.

The DPPH radical scavenging assay of the extracts as represented in Table 3 showed that, the scavenging activities of all the crude extracts on DPPH were relatively similar. Although ethyl acetate and acetone at 50µg/ml and 300µg/ml respectively showed the same activity with the standard sample (Vit C). The ABTS scavenging activity revealed that, from 150µg/ml, ethyl acetate (19.72±0.00) and aqueous (18.50±0.01) crude extracts showed better scavenging activity compared to other extracts with the exception of the standard. The mechanism involved in the scavenging activity of these samples may be attributed to their phenol contents (Moukette *et al*., 2015). The FRAP and NO assays also showed a relatively similar antioxidant activity, while Vitamin C used as standard showed increasing activity in a dose dependent manner (Moukette *et al*., 2015).

It was found from the OH radical scavenging assay that, the extracts showed inhibitory potential against OH, but at the lowest concentration (25µg/ml), specifically, ethyl acetate showed high scavenging potential (65.53±0.04) than Vitamin C (53.76±0.01). However, with increase concentration, ethyl acetate, chloroform, methanol and water extracts exhibited the same scavenging potential with vitamin C at $P<0.05$.

Different values of fifty percent inhibitory concentration (IC_{50}) of the tested extracts were calculated and ethyl acetate which generally demonstrated the highest percentage inhibition, showed the lowest IC_{50} for ABTS (268.49 ± 0.02), NO (49.81 ± 0.04) and OH (58.43 ± 0.64), and this agreed with the assertion that, the IC_{50} of the plant samples is inversely proportional to its antioxidant power due to its polyphenol content (Moukette *et al*., 2015). Although according to Gubler *et al*., (2013), a good correlation is not always observed between them.

The results (Table 3) showed that the scavenging activity of all the crude extracts on DPPH assay is generally the same at varying concentration, though ethyl acetate and acetone at 50µg/ml and 300µg/ml respectively showed the same activity with the standard sample (Vit. C). Thus, the results of these antioxidant activities may not be dose dependent.

Table 3 DPPH Radical Scavenging Activity of Crude Extracts of *P. amarus* Leaves

	Conc. Sample (µg/ml)				
	25	**50**	**75**	**150**	**300**
Extract	**DPPH Inhibition (%)**				
Hexane	26.26±0.02^{ae}	31.05±0.00^{ae}	31.54±0.02^{ae}	31.77±0.01^{ae}	34.92±0.03^{ae}
Chloroform	24.55±0.01^{ae}	27.09±0.03^{ae}	28.75±0.00^{ae}	28.83±0.03^{ae}	29.48±0.04^{ae}
Ethyl acetate	31.25±0.01^{ae}	31.85±0.01^{ce}	33.42±0.02^{ae}	33.79±0.03^{ae}	33.99±0.01^{ae}
Acetone	0.88±0.02^{a}	9.19±0.05^{a}	20.01±0.03^{a}	33.03±0.03^{ae}	48.93±0.04^{ce}
Methanol	28.66±0.03^{ae}	28.36±0.08^{ae}	31.05±0.02^{ae}	40.54±0.13^{ae}	44.58±0.01^{ae}
Aqueous	22.03±0.06^{ae}	29.23±0.05^{ae}	31.38±0.03^{ae}	35.23±0.03^{ae}	39.07±0.05^{ae}
Vit.C (Standard)	47.32±0.03^{b}	49.91±0.04^{b}	68.86±0.05^{b}	70.67±0.02^{b}	72.31±0.04^{b}

Values are expressed as mean ±SD of three replicates. ^{b} Significant at p=0.05 compared to ^{a} but not significant to ^{c}. ^{e,} has same activity within the column.

The results (Table 4) showed that, from 150µg/ml and above, ethyl acetate and aqueous crude extracts showed better scavenging activity compared to other extracts.

Table 4 ABTS Scavenging Potential of Crude extracts of *P. amarus* Leaves

	Conc. sample (µg/ml)				
	25	**50**	**75**	**150**	**300**
Extract	**ABTS Inhibition (%)**				
Hexane	7.86±0.01^{ae}	8.91±0.02^{ae}	7.74±0.02$^{a•}$	14.25±0.00$^{a•}$	17.79±0.02$^{e•}$
Chloroform	4.29±0.03^{ae}	4.96±0.00^{ae}	6.33±0.01$^{a•}$	6.33±0.00^{a}	13.66±0.00$^{e•}$
Ethyl acetate	10.59±0.02^{ae}	12.97±0.04^{ae}	14.49±0.01^{ae}	19.72±0.00^{ae}	39.31±0.03^{ae}
Acetone	1.79±0.02^{ae}	8.59±0.04^{ae}	8.96±0.02^{ae}	15.47±0.02^{ae}	21.15±0.01$^{e•}$
Methanol	8.51±0.02^{ae}	9.89±0.02^{ae}	11.76±0.01^{ae}	10.35±0.01$^{a•}$	19.61±0.02$^{e•}$
Aqueous	10.81±0.02^{ae}	13.69±0.02^{ae}	18.66±0.01^{af}	18.50±0.01^{ae}	36.99±0.02^{ae}
Vit.C (Standard)	40.98±0.03^{b}	69.64±0.01^{b}	93.42±0.01^{b}	94.91±0.00^{b}	94.68±0.00^{b}

Values are expressed as mean ±SD of three replicates. ^{b} Significant at p=0.05 compared to ^{a}. ^{e,} $^{•}$ has same activity. ^{f} Significant (P=0.05) compared to $^{e•}$ within the column.

The results (Table 5) showed that the antioxidant potential of all the different crude extracts have the same scavenging potential for nitric acid radical ($NO^{•}$), including the standard sample at P<0.05.

Table 5 NO Scavenging Potential of Crude Extracts of *P. amarus* Leaves

	Conc. Sample (µg/ml)				
	25	**50**	**75**	**150**	**300**
Extract	**NO Inhibition (%)**				
Hexane	78.22±0.05^{a}	74.50±0.07^{a}	74.04±0.08^{a}	69.86±0.10^{a}	60.19±0.16^{a}
Chloroform	70.39±0.01^{a}	70.94±0.16^{a}	76.10±0.10^{a}	78.06±0.04^{a}	82.09±0.14^{a}
Ethyl acetate	71.95±0.03^{a}	73.42±0.02^{a}	77.15±0.03^{a}	78.73±0.07^{a}	81.26±0.01^{a}
Acetone	65.18±0.13^{a}	66.31±0.13^{a}	68.13±0.06^{a}	71.44±0.12^{a}	74.90±0.04^{a}
Methanol	63.59±0.03^{a}	67.07±0.16^{a}	69.67±0.03^{a}	69.65±0.03^{b}	74.97±0.08^{a}
Aqueous	69.58±0.10^{a}	73.80±0.12^{a}	74.31±0.12^{a}	75.83±0.16^{a}	78.02±0.09^{a}
Vit.C (Standard)	74.59±0.07^{a}	76.22±0.08^{a}	80.76±0.07^{a}	84.39±0.06^{a}	86.98±0.05^{a}

Values are expressed as mean ±SD of three replicates. ^{a} has same activity at P= 0.05 within the column.

The results of OH scavenging potential of extracts of *Phyllanthus amarus* are present in Table 6. The results showed that at 25µg/ml, ethyl acetate showed high scavenging potential than Vitamin C. However, with increase concentrations, ethyl acetate, chloroform, methanol and aqueous extracts exhibited the same scavenging potential with vitamin C at P <0.05.

Table 6 Hydroxyl (OH) Scavenging Potential of Crude Extract of *P. amarus* Leaves

	Conc. Sample (µg/ml)				
	25	**50**	**75**	**150**	**300**
Extract	**OH Inhibition (%)**				
Hexane	64.52±0.06^{c}	58.47±0.09^{c}	57.71±0.11^{a}	50.90±0.13^{a}	35.16±0.21^{a}
Chloroform	67.16±0.01^{c}	69.70±0.02^{c}	69.87±0.01^{ce}	71.36±0.02^{ce}	72.12±0.03^{ce}
Ethyl acetate	65.53±0.04^{b}	69.76±0.03^{b}	70.45±0.03^{be}	71.46±0.04^{be}	73.58±0.01^{be}
Acetone	42.01±0.08^{a}	47.71±0.09^{a}	54.57±0.02^{a}	54.78±0.03^{a}	54.78±0.02^{a}
Methanol	52.19±0.04^{ae}	56.28±0.03^{c}	58.90±0.02^{a}	70.83±0.03^{ce}	74.53±0.06^{ce}
Aqueous	52.99±0.07^{ae}	55.92±0.15^{c}	70.32±0.02^{ce}	70.33±0.04^{ce}	71.95±0.05^{ce}
Vit. C (Standard)	53.76±0.01^{ae}	65.43±0.00^{c}	68.67±0.09^{ce}	74.58±0.08^{ce}	78.79±0.07^{ce}

Values are expressed as mean ±SD of three replicates. ^{b} Significant at p=0.05 compared to ^{a} but no significant to ^{c}. ^{e} has same activity within the column.

According to Table 7, it appeared that all the crude extracts showed relatively similar antioxidant activities at varying concentrations at P=0.05. Vitamin C used as standard showed increasing activity in a dose dependent manner.

Table 7 FRAP Antioxidant Potential of Crude Extracts of *P. amarus* Leaves

	Conc. Sample(µg/ml)				
	25	**50**	**75**	**150**	**300**
Extract	**FRAP Inhibition (%)**				
Hexane	6.35±0.00^{a}	6.45±0.00^{a}	6.31±0.00^{a}	7.61±0.00^{a}	8.94±0.01^{a}
Chloroform	6.62±0.01^{a}	6.87±0.01^{a}	6.04±0.00^{a}	6.98±0.01^{a}	7.68±0.01^{a}
Ethyl acetate	6.18±0.00^{a}	6.44±0.00^{a}	6.31±0.01^{a}	7.14±0.00^{a}	7.19±0.01^{a}
Acetone	6.06±0.00^{a}	6.11±0.01^{a}	6.29±0.01^{a}	6.86±0.00^{a}	8.82±0.01^{a}
Methanol	7.15±0.02^{a}	6.87±0.02^{a}	7.74±0.03^{a}	8.34±0.04^{a}	10.07±0.03^{a}
Aqueous	8.09±0.03^{a}	7.47±0.04^{a}	7.59±0.04^{a}	7.95±0.03^{a}	9.12±0.03^{a}
Vit. C (Standard)	23.16±0.02^{b}	36.12±0.04^{b}	55.33±0.06^{b}	82.89±0.06^{b}	114.55±0.09^{b}

Values are expressed as mean ±SD of three replicates. ^{b} Significant at p=0.05 compared to ^{a} . ^{a} has same activity within the column.

IC_{50} of Crude Extracts on the Radicals Tested

As given in Table 8, the result demonstrated that ethyl acetate displayed the lowest IC_{50} against ABTS, NO, OH radicals while methanol showed the lowest IC_{50} for DPPH and FRAP beside the standard (Vit.C)**.**

Table 8 IC_{50} of Crude Extracts on the Radicals Tested

	IC_{50} (µg/ml)				
Extract	**DPPH**	**ABTS**	**FRAP**	**NO**	**OH**
Hexane	175.85 ± 0.78^{a}	510.71 ± 0.71^{b}	903.76 ± 0.44^{c}	57.38 ± 0.71^{d}	96.84 ± 0.14^{e}
Chloroform	201.80 ± 0.02^{a}	855.48 ± 0.28^{b}	957.54 ± 1.42^{c}	50.94 ± 0.07^{d}	59.49 ± 0.08^{e}
Acetone	219.82 ± 0.04^{a}	505.73 ± 0.02^{b}	938.44 ± 0.14^{c}	60.39 ± 0.02^{d}	94.43 ± 0.03^{e}
Ethyl acetate	167.69 ± 0.28^{a}	268.49 ± 0.02^{b}	1029.78 ±13.66^{c}	49.81 ± 0.04^{d}	58.43 ± 0.64^{e}
Methanol	149.84 ± 1.17^{a}	487.80 ± 0.71^{b}	796.56 ± 2.89^{c}	60.15 ± 0.07^{d}	69.19 ± 0.01^{e}
Aqueous	171.09 ± 0.44^{a}	276.63 ± 0.71^{b}	817.40 ± 0.71^{c}	53.23 ± 0.70^{d}	67.17 ± 0.04^{e}
Vit. C (Standard)	69.84 ± 0.02^{a}	40.00 ± 0.01^{b}	65.35 ± 0.14^{c}	44.13 ± 0.01^{d}	60.69 ± 0.20^{e}

Values are expressed as mean ± SD of three replicates. Values with same superscripts in the same column are significantly different at P<0.05.

Pearson Correlation between Polyphenol Contents and Antioxidant Power of Crude Extracts of *P amarus* Leaves

The correlation between the polyphenols and antioxidant power of the extracts under study are given in Tables 9, 10, 11, 12, 13 and 14.

The correlation between the free radical scavenging activity, antioxidant power assays and the polyphenol content were also studied for each extract using Pearson's correlation analysis and the results were presented in Tables 9-14. It was found that, ethyl acetate extract showed positive and significant correlation between ON, ABTS, FRAP radicals to phenols with coefficient of 0.993, 0.996, and 0.920 respectively while OH radical to flavonoids and tannins (0.875, 0.866). DPPH to

tannins has a coefficient of 0.854. An evaluation on the correlations of each extract with their polyphenol content revealed that ethyl acetate was the best. This agreed with pervious finding by Soumya *et al*., (2013) and Sushant *et al*., (2019) that the richer the polyphenol content, the better the antioxidant potential of the plant. The hydroxyl (OH^*) radicals are the most reactive chemical species and potent cytotoxic agent which attack and cause damage to every molecule found in living tissues (Anbudhasan *et al*., 2014). Thus, the strong positive correlation of the flavonoids and tannins contents of ethyl acetate extract of *P. amarus* leaves to OH^* radicals strongly suggest its usefulness as source to remedy many cytotoxic diseases like cancer, aging, heart diseases, reduce oxidative stress and immune infections.

Table 9 shows the correlation between polyphenols and antioxidant power for hexane crude extract. The analysis revealed a very weak correlation.

Table 9 Correlation between Polyphenols and Antioxidant Power of Hexane Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANNINS	0	1						
PHENOLIC ACIDS	0	0	1					
DPPH	0.5	0	0	1				
ABTS	-0.81706	0	0	-0.90784	1			
NO	0.5	0	0	1	-0.90784	1		
OH	0	0	0	0.866025	-0.57656	0.866025	1	
FRAP	-1	0	0	-0.5	0.817057	-0.5	0	1

Significant values p=0.05 (bilateral test)

The result of table 10 shows the correlation of polyphenols and antioxidant power for chloroform crude extract. There is no strong correlation between its polyphenol contents and its antioxidant power except for flavonoids and tannins to ABTS which showed strong positive correlation with coefficients of 0.866025404 and 0.944911183 respectively.

Table 10 Correlation between Polyphenols and Antioxidant Power of Chloroform Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANNINS	0.654653671	1						
PHENOLIC ACIDS	0	0	1					
DPPH	-0.72057669	0.052414242	0	1				
ABTS	0.866025404*	0.944911183*	0	-0.27735	1			
NO	-0.5	0.327326835	0	0.960769	-0.25113	1		
OH	-0.5	-0.98198051	0	-0.24019	-0.86603	-0.5	1	
FRAP	-0.32732684	0.5	0	0.891042	0.188982	0.981981	-0.65465	1

Significant values p=0.05 (bilateral test)

In Table 11 the flavonoids content of the extract showed strong positive correlation to ABTS, OH, and FRAP radicals whereas, phenolic acids showed strong positive correlation to ON, OH, DPPH and FRAP respectively as indicated by asterisks (*).

Table 11 Correlation between Polyphenols and Antioxidant Power of Acetone Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANNINS	-0.5	1						
PHENOLIC ACIDS	0	-0.8660254	1					
DPPH	0.5	0.5	-0.86603	1				
ABTS	0.981980506*	-0.65465367	0.188982	0.327327	1			
NO	0.5	-1	0.866025*	-0.5	0.654654	1		
OH	1*	-0.5	-0.24113	0.5	0.981981	0.5	1	
FRAP	1*	-0.5	0	0.5	0.981981	0.5	1	1

Significant values p=0.05 (bilateral test)

From the ethyl acetate crude extract in Table 12, the analysis revealed a positive and strong significant correlation between its phenolic content and its antioxidant activity. For instance; flavonoids and tannins have a coefficient of 0.844280264 and 0.8536185 with DPPH respectively and 0.874725789 and 0.8660254 with OH respectively. Phenolic acids have a coefficient of 0.9965407, 0.9933993, and 0.9201931 with ABTS, ON, FRAP respectively.

Table 12 Correlation between Polyphenols and Antioxidant Power of Ethyl Acetate Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANINS	0.999843852	1						
PHENOLIC ACIDS	-0.85705456	-0.866025	1					
DPPH	0.844280264*	0.8536185*	-0.999705	1				
ABTS	-0.8112711	-0.821476	0.9965407*	-0.998266	1			
NO	-0.91049781	-0.917663	0.9933993*	-0.990318	0.9804298	1		
OH	0.874725789*	0.8660254*	-0.5	0.4788059	-0.426298	-0.59604	1	
FRAP	-0.58696293	-0.601178	0.9201931*	-0.929435	0.9495431	0.869215	- 0.121078	1

Significant values p=0.05 (bilateral test)

Table 13 revealed the correlation of polyphenols contents and antioxidant power of methanol crude extract. Only flavonoids showed positive correlation to its antioxidant power. Flavonoids has significant correlation to NO and FRAP.

Table 13 Correlation between Polyphenols and Antioxidant Power of Methanol Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANNINS	-0.0822	1						
PHENOLIC ACIDS	-0.56949	0.866025	1					
DPPH	-0.9979	0.017493	0.515073	1				
ABTS	0.492519	-0.90784	-0.99587	-0.43513	1			
NO	0.821995*	0.5	-2.12113	-0.85715	-0.09078	1		
OH	0.082199	-1	-0.86603	-0.01749	0.907841	-0.5	1	
FRAP	0.984853*	0.091849	-0.41834	-0.99401	0.334157	0.908289	-0.09185	1

Significant values p=0.05 (bilateral test)

Table 14 reveals the correlation between polyphenols contents and antioxidant power for aqueous crude extract of *P. amarus* leaves. Only tannins showed positive correlation to its antioxidant power. It has a significant correlation to DPPH and NO.

Table 14 Correlation between Polyphenols and Antioxidant Power of Aqueous Crude Extract of *P. amarus* Leaves

	FLAVONOIDS	TANNINS	PHENOLIC ACIDS	DPPH	ABTS	NO	OH	FRAP
FLAVONOIDS	1							
TANNINS	-0.98198	1						
PHENOLIC ACIDS	0.785714	-0.65465	1					
DPPH	-0.98198	1*	-0.65465	1				
ABTS	-0.32733	0.5	0.327327	0.5	1			
NO	-0.75593	0.866025*	-0.18898	0.866025	0.866025	1		
OH	0.188982	0	0.755929	0	0.866025	0.5	1	
FRAP	0.755929	-0.86603	0.188982	-0.86603	-0.86603	-1	-0.5	1

Significant values p=0.05 (bilateral test)

CONCLUSION

Conclusively, this study found that the richer the polyphenol content, the better the antioxidant potential of the extracts of *P. amarus* leaves and ethyl acetate is the best solvent for extraction of polyphenols to address diseases relating to oxidative stress. There is a strong positive correlation between its polyphenol content and its antioxidant potential.

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